REMARKS

Claims 26, 27, 29-34, 41-44, and 46 are pending in this application. Claims 26, 27, 29-34, 41-44, and 46 stand rejected. No claims stand objected to. The Applicants herein amend Claims 26, 27, 20-35, 41-44, and 46 to clarify the scope of the instantly claimed invention. These claim amendments find support in the original claims. The Applicants also herein add new Claims 51-56. New Claims 51 and 52 find support at page 5, line 30 through page 6, line 9 of the as-filed specification, and in the as-filed claims. New Claim 53 finds support at pages 5, lines 8-10 and line 30, and page 9, lines 12-23, as well as elsewhere in the as-filed specification. New Claims 54 and 55 find support in the as-filed claims, as well as in the specification from page 2, line 20 through page 3, line 26. New Claim 56 finds support in the as-filed claims, as well at in the as-filed specification at page 5, line 30 through page 6, line 23. Therefore, none of these claim amendments raise issues of new matter.

At the outset, the Applicants wish to thank the Examiner for the courtesy she showed the Applicant's undersigned attorney, Dr. Crepin, and Dr. Neumeier in the interview on February 28, 2006 (herein "the interview"). First, the interview entailed a discussion of the outstanding section 112, second paragraph rejections. The Examiner agreed that, if the Applicants deleted the portion of those claims that related to multiple dosing, and added new method and/or kit claims to cover these embodiments, they could overcome this rejection without violating the Restriction Requirement dated June 20, 2003.

Next, the interview turned to the various rejections under 35 U.S.C. § 103 that the Examiner made in the Final Office Action. During that discussion, Dr. Neumeier explained to the Examiner the difference between epidemic, also known as seasonal flu, and pandemic flu; this distinction pertains to whether the population is primed, as in an epidemic, or unprimed, as in a pandemic. At that time, Dr. Neumeier wrote down on a blank sheet of paper the dates of all of the 20th century pandemics and their associated flu virus subtypes. The Examiner asked to retain this sheet as an exhibit, which is referred to in the Interview Summary, and the Applicants agreed to leave it with her after the interview. That sheet of paper exhibit is the sole exhibit that was discussed in the interview. After Dr. Neumeier explained how developing a flu vaccine for an epidemic differs from doing so for a pandemic, the interview turned to the five cited pieces of art from the Final Office Action.

Subsequently, the Examiner mentioned that she did an internet search on pandemic flu the morning of the interview. In conjunction with this search, she indicated that she found the following three documents, copies of which she supplied to the Applicants' undersigned attorney during the interview, that she contended could be relevant to the instant application: (1) Enserink, *Science* 310: 1889 (Dec. 23, 2005) (copy enclosed); (2) http://www.newscientist.com/channel/halth/bird-flu/dn8478 (Dec. 16, 2005) (copy enclosed); and (3) "WHO Meeting on Development and Evaluation of Influenza Pandemic Vaccines, Sanofi Pasteur, France—H5N1 Vaccine, Nov. 2-3, 2005", www.who.int/entity/vaccine_research/diseases/influenza/gerdil.pdf (downloaded Feb. 28, 2006) (copy enclosed). None of these three pieces of art constitutes prior art to the instant application, and the Examiner indicated that she had yet not decided whether she would make a rejection based upon any of this newly discovered art. As the Interview Summary reflects, none of these three pieces of art contains data on aluminium-adjuvanted H5N1 pandemic vaccine in humans; the Examiner further indicated that, should such data become available during the prosecution of the instant application, it could become relevant to the enablement of the H5N1 dependent claims in the instant application.

CLAIM REJECTIONS UNDER 35 U.S.C. § 112

Claims 26-35, 41-44, and 46 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Specifically, the Examiner contends that the phrase, "combined dose" in these claims fails to define the metes and bounds of the composition that Applicants intend to claim. During the interview, the Examiner suggested that the Applicants break out the portions of these rejected claims that relate multiple doses into method and/or kit claims, and that the introduction of such new claims would not violate the Restriction Requirement dated June 20, 2003.

In response to these rejections, the Applicants herein amend Claims 26-35, 41-44, and 46 to remove the recitation of "combined dose". In addition, the Applicants herein add new Claims 51 and 52, which recite methods of treatment with the instantly claimed vaccine composition, as well as new Claim 56, which recites a kit comprising the same. In view of the Examiner's statements during the Interview, the Applicants submit that these claim amendments render these rejections moot. Accordingly, the Applicants respectfully request reconsideration and withdrawal of the rejections of Claims 26-35, 41-44, and 46 under 35 U.S.C. § 112, second paragraph.

CLAIM REJECTIONS UNDER 35 U.S.C. § 103

Claims 26, 27, 29-34, 41-44, and 46 stand rejected under 35 U.S.C. § 103 as allegedly unpatentable over the combined teachings of Couch, *et al.*, (*J. Infect. Dis.*, 176: S38-S44 (1997)), Chaloupka, *et al.*, (*Eur. J. Clin. Microbiol. & Infect. Dis.*, 15: 121-127 (1996)), and either or both of Schenk, *et al.*, (*Pharmatherapeutica*, 3(3): 201-308 (1982)), and Pressler, *et al.*, (*Pharmatherapeutica*, 3(3): 195-200 (1982)). The Examiner argues that Couch, *et al.* "explicitly suggests developing adjuvants to produce satisfactory immune responses with lower doses of antigens to decrease the burden of vaccine production in a pandemic circumstance." *See* the sentence bridging pages 5 and 6 of the Office Action dated February 11, 2004. She opines that Chaloupka, *et al.* teach that current influenza vaccines are required to contain 15 μg hemaglutinin per dose. The Examiner further alleges that Schenk, *et al.* and Pressler, *et al.* both teach that the aluminium-adjuvanted vaccine is superior in persons without previous immunity to the influenza strain used. She then concludes that, "in order to carry out the suggestion of Couch to produce satisfactory immune response with lower doses of antigen to decrease the burden of vaccine production in a pandemic circumstance, it would have been within the ordinary skill of the art to choose an aluminium-based adjuvant, with a reasonable expectation of success." *See* Final Office Action at page 3.

The Applicants respectfully traverse these rejections. For a proper obviousness rejection under section 103, the Examiner has the burden of establishing, *prima facie,* with evidence or reasons that, *inter alia*, at the time of the invention: (1) the prior art of record would have motivated one of ordinary skill in the art to make or carry out the combination and modification of the prior art as suggested by the Examiner to arrive at the claimed invention; and (2) "the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have had a reasonable expectation of success. Both the suggestion [or motivation] and the reasonable expectation of success must be founded in the prior art, not in [Applicants'] disclosure." *In re Vaeck*, 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991) (citations omitted).

During the interview, Dr. Neumeier elucidated the differences between developing a flu vaccine for epidemic influenza strains to be used in a primed population, versus doing so for a pandemic influenza strain to be used in an unprimed population. She listed the three major pandemics of the 20th century with their HN types as follows: 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2). Dr. Neumeier stressed that anyone born after 1968, the last flu

pandemic, would be unprimed with regard to the H2N2 subtype. Persons born before 1968 would be considered comprise the primed population. A pandemic is defined as a situation wherein a substantial portion of the world population is unprimed. When referring to the cited art, the Applicants submit that it is important to distinguish between teachings in a primed versus unprimed population; one cannot conclude that an unprimed population will have the same strength and duration of response to a flu vaccine then will a primed population.

Schenk, *et al.* reports data from unprimed (24 years or less) versus primed (over 24 years) subjects with six monovalent flu vaccines, three of which are fluid, and three of which are adsorbed. During the interview, Dr. Neumeier explained the relevance of this primed/unprimed distinction with respect to Table 2 of Schenk, *et al.* (page 205). As of the 1982 publishing date of Schenk, *et al.*, subjects over 24 years old were primed, while those under 24 years old were unprimed with respect to the vaccine antigen A/New Jersey/8/76 (Hsw1N1). Table 2 shows HAI antibody titres (reciprocal values) at increasing dose ranges over time. At day 30, for example, the adsorbed vaccine in the younger population (24 years or less) gave the following results: 86.4 (54 IU/dose), 259.9 (108 IU/dose), and 160.0 GMT (216 IU/dose). At the same time point (day 30), the primed population (over 24 years) shows dramatically different results for the adjuvanted vaccine tested: 570.0 (54 IU/dose), 211.1 (108 IU/dose), and 735.2 GMT (216 IU/dose). These starkly contrasting data from Table 2 of Schenk, *et al.* powerfully illustrate Dr. Neumeier's distinction regarding the primed versus unprimed immune status of a population when formulating a flu vaccine. The Applicants assert that the results of Table 2 would not have motivated the skilled artisan to choose the low dose. In fact, the Applicants submit that Table 2 of Schenk, *et al.* teaches away from lowering the dose of antigen in an unprimed population.

The Applicants also point out that Schenk, *et al.* does not use the same way to quantify the HA as do the Applicants in the instant application. The amounts of antigen used in Schenk, *et al.* are standardized by HA assay which is then translated to International Units (I.U.). In the application single-radial-diffusion (SRD) method is used to quantify and standardize the HA content as μg/dose; From the data in Schenk *et al.*, it is impossible to extrapolate or bridge the two different measurement units. To buttress this argument, the Applicants enclose Wood, *et al.*, *J. of Biological Standardization*, 5: 237-247 (1977). The Applicants direct the Examiner's attention to the second and third paragraphs on page 237 of

Wood, *et al.* Therefore, the skilled artisan would be wholly unable to ascertain what dose Schenk, *et al.* employed.

For all of these reasons, the Applicants contend that Schenk, *et al.* neither teaches nor suggests the instantly claimed invention with a reasonable expectation of success. In fact, in view of the points that the Applicants made above with respect to primed versus unprimed populations, low dose, and adjuvants, they submit that Schenk, *et al.* teaches away from the instantly claimed invention. A reference should be considered as a whole, and portions arguing against or teaching away from the claimed invention must be considered. *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve, Inc.*, 230 U.S.P.Q. 416, 419 (Fed. Cir. 1986).

Couch, *et al.*, a review article, which was published 15 years after Schenk, *et al.*, but does not cite to Schenk, *et al.*, only contains data on an epidemic flu vaccine (*i.e.*, a primed population). Couch, *et al.* teaches failures with newer, more complex adjuvants (*see* S40 and S42). S40 indicates: "Thus, addition of a number of newer adjuvants to IVVs has not yet led to increases in serum antibody responses comparable to those reported decades ago." Aluminium, which the instant claims recite, is an older and simpler adjuvant. The Applicants submit that Couch, *et al.* also teaches away from the instantly claimed invention, because it teaches increasing the dose of antigen to increase magnitude and duration of response. *See* the last sentence of the abstract: "Increasing the doses of hemagglutinin and neuraminidase, using adjuvants or immunomodulators, and administering IVVs by the mucosal route could improve performance of the vaccines." By contrast, the instant claims recite only low doses of vaccine, *i.e.*, no more than 15 μg. Also, the only mention that Couch, *et al.*, makes about an alum adjuvant is in a historical sense: "Adjuvants substantially enhanced serum HAI antibody responses of vaccines used during the 1940s and 1950s; both alum and water in oil emulsions were used . . . ." *See* S40. As Dr. Neumeier noted, during this time period, the population would have been primed for the antigens contained in the vaccines used during the 1940s and 1950s.

Moreover, the few instances where Couch, *et al.* raises the issue of developing a pandemic flu vaccine, it only does so in an entirely tentative way. For example, S41 of Couch, *et al.* provides: "Adjuvants **could** be of particular value in pandemic circumstances either for the large number of persons who would be primed or as a means of conserving antigen that is available in limited quantities." (emphasis added). In addition, Couch, *et al,* at S42 states: "Thus IVVs **can** prevent pandemic influenza, but **there is uncertainty** about which IVV characteristics are necessary and the vaccination time frame. . ." (emphasis

added). In addition, on S42 under "Final Comments", Couch, *et al.* state: "Moreover, there is experience available from prior pandemics suggesting that IVVs **may** be even less effective in pandemic situations than in interpandemic epidemic outbreaks." (emphasis added).

The Applicants submit that these speculative statements in Couch, *et al.* provide no motivation to the skilled artisan to combine its teachings with Schenk, *et al.* to arrive at the instantly claimed invention. At most, Couch, *et al.* calls out for further research into the pandemic flu vaccine production field. It is improper to reject claims as "obvious to try" where the motivation to combine references arises merely because the subject matter of the claimed invention is a promising field for experimentation, although the prior art provides only general guidance as to particular form of the claimed invention or how to achieve it. *In re O'Farrell*, 7 U.S.P.Q.2d 1673, 1681 (Fed. Cir. 1988).

Chaloupka, *et al.*, a review article that characterizes six commercially available, inactivated, trivalent vaccines, contributes only the standard dose of flu vaccine of 15 μg. Otherwise, the Applicants respectfully submit that Chaloupka, *et al.* is totally irrelevant to the instantly claimed subject matter; it makes no mention of adjuvants or pandemic, whatsoever, not does it suggest developing improved flu vaccine formulations. Moreover, the Applicants acknowledge, at page 4, lines 26-27 of the as-filed specification, that they consider 15 μg of vaccine to be a regular dose.

Pressler, *et al.* use a low dose, bivalent vaccine in a primed population (vaccine components A/Victoria/3/75 and B/Hong Kong/8/73 that circulated 3 and 5 years before study start). By contrast, the Applicants claim a monovalent, low dose vaccine to be used in an unprimed population. The only common link between the instant application and Pressler, *et al.* is the low dose. As the Applicants argue above with respect to Schenk, *et al.* a primed population is not relevant to the instantly claimed invention; by definition, a population in a pandemic is unprimed, as discussed above.

In view of the foregoing arguments, the Applicants respectfully submit that Schenk, *et al.*, Chaloupka, *et al.*, Couch, *et al.*, and Pressler, *et al.*, neither alone, nor in combination, would have motivated the skilled artisan to arrive at the instantly claimed vaccine with any reasonable expectation of success. Although the prior art could be readily modified to form the claimed invention, "[t]he mere fact that the prior art could be so modified would not have made the modification obvious unless the prior art suggested the desirability of the modification." *In re Laskowski,* 10 U.S.P.Q.2d 1397, 1398-99 (Fed. Cir. 1989).

Furthermore, the Applicants submit that the only way that one could arrive at their invention from the cited art is through engaging in impermissible hindsight reconstruction. "It is impermissible . . . simply to engage in a hindsight reconstruction of the claimed invention, using the applicant's structure as a template and selecting elements from references to fill in the gaps." *In re Gorman*, 18 U.S.P.Q.2d 1885, 1888 (Fed. Cir. 1991) (*citing Interconnect Planning Corp. v. Feil*, 227 U.S.P.Q. 543, 551 (Fed. Cir. 1985)).

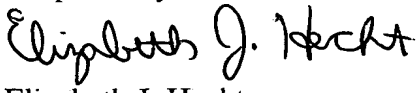
Claims 44 and 46 stand rejected under 35 U.S.C. § 103 as allegedly unpatentable over the combined teachings of Couch, *et al.*, Chaloupka, *et al.*, and either or both of Schenk, *et al.*, and Pressler, *et al.* as applied to Claims 26, 27, 29-34, 41-44, and 46, further in view of Riberdy, *et al.*, *J. Virol.*, 73: 1453-1459 (1999). For the reasons argued above, the Applicants contend that Couch, *et al.*, Chaloupka, *et al.*, Schenk, *et al.*, and Pressler, *et al.* fail to teach or suggest the instantly claimed invention. Moreover, the Applicants agree with the Examiner's conclusion about the teachings of Riberdy, *et al.*, *i.e.*, that skilled artisans believe that the H5N1 virus has pandemic potential and is being used to develop a vaccine for humans. That said, the Applicants submit that Riberdy, *et al.* has no relevance to the instant claims. Nowhere does Riberdy, *et al.* mention the use of an adjuvanted vaccine for a pandemic.

In summary, none of the five cited references, either separately or in combination, teach or suggest the claimed invention with a reasonable expectation of success. Therefore, because none of the obviousness rejections in the Final Office Action fulfills either requisite prong of *In re Vaeck*, no *prima facie* case of obviousness exists. The Applicants submit their arguments have overcome all of the obviousness rejections in the Final Office Action. Accordingly, they respectfully request reconsideration and withdrawal of all of the rejections of Claims 26, 27, 29-35, 41-44, and 46 under 35 U.S.C. § 103.

The Applicants thank the Examiner for the Final Office Action, and believe that they have responded fully to it. In view of the foregoing amendments and remarks, the Applicants respectfully submit that the subject application is in condition for allowance. If the Examiner

has any remaining objections or concerns, the Applicants respectfully request her to contact their undersigned attorney to resolve such issues and advance this application to issue.

Respectfully submitted,



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Tests dash hopes of rapid production of bird flu vaccine

17:43 16 December 2005
NewScientist.com news service
Debora MacKenzie

The results of first large-scale trials of a low-dose vaccine against H5N1 bird flu have been announced – and they are unexpectedly disappointing. Scientists had hoped that very low doses of vaccine virus would make humans immune if injected along with an immune-stimulating chemical called an adjuvant.

But on Thursday, French vaccine company Sanofi pasteur announced that in tests on 300 people in France, they did not. "The prospects for adequate global supplies of an effective pandemic vaccine of any kind are dimmer now than they were last week," David Fedson, founder of the vaccine industry's pandemic task force, told **New Scientist**.

The first tests of H5N1 vaccine in the US in August 2005 found that the virus on its own does not stimulate much immunity in people. To elicit enough to ward off disease, a vaccine required 90 micrograms of the virus's main surface protein - six times more than is needed in ordinary flu vaccine.

The less virus is required per dose of vaccine, the more doses vaccine factories can produce in the limited time that will be available to immunise people at the start of a pandemic. Research with other types of bird flu have found such low-dose vaccines are possible, if the virus is combined with an adjuvant.

Antibody response

So Sanofi tested various doses of a vaccine virus based on the H5N1 that killed people in Vietnam in 2004, plus the most widely used adjuvant, alum. But no dose under 30 micrograms elicited enough antibodies to meet official standards for flu vaccines.

This means, says Fedson, that "if all the world's influenza vaccine companies were to produce this vaccine for six months, there would be enough to vaccinate only 225 million people".

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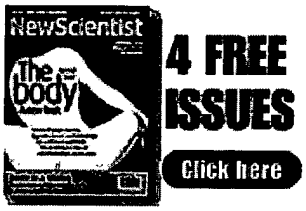

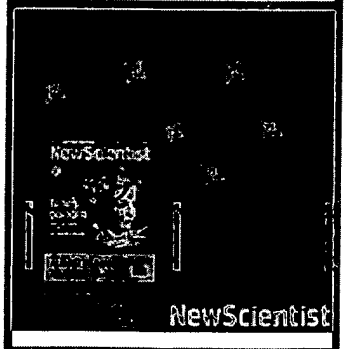

"Thirty micrograms is obviously too high," says Agnes Hoffenbach, Sanofi's head of pandemic vaccine research. "In our next studies we will try and get lower doses to work."

One problem may be the adjuvant. The earlier studies of other types of bird flu used one called MF59, patented by the US firm Chiron. Supplies are limited and it is not yet approved for human use in the US and some other countries.

Split virus

Another problem could be that Sanofi's vaccine contained a split virus, rather than whole, killed virus. Split viruses are used for standard flu vaccines, as they elicit fewer side effects, but whole viruses are suspected to be more immunogenic.

China's Sinovac company is now testing a whole-virus H5N1 vaccine with alum. Takato Odagiri, head of the flu virus lab at Japan's National Institute of Infectious Diseases, reported at an international meeting in Malta in September that in his tests, w seemed more immunogenic than split virus. He is now focusing his research solely on v

But Hoffenbach told **New Scientist**: "I don't think we will look at whole virus vaccines." manufacturing processes that would have to be tested and approved. "We used our exi the fastest way to move to large-scale production." Tests of Sanofi H5N1 vaccine plann also use split virus.

Fedson says more avenues should be tried. "The prospects for developing a pandemic produced in the quantities the world will demand are now enormously more difficult," he more work to do."

Bird flu: A New Scientist special report

A flu pandemic could kill billions of people. Become an **Instant Expert** and read all the l special report

Mammoth achievement. Researchers managed to sequence a large chunk of DNA from a Siberian mammoth.

CREDIT: GIANNI DAGLI ORTI/CORBIS

ANCIENT DNA

New Methods Yield Mammoth Samples

Ancient DNA has always held the promise of a visit to a long-vanished world of extinct animals, plants, and even humans. But although researchers have sequenced short bits of ancient DNA from organisms including potatoes, cave bears, and even Neandertals, most samples have been too damaged or contaminated for meaningful results.

Now in a paper published online by *Science* this week*, an international team reports using new technology to sequence a staggering 13 million basepairs of both nuclear and mitochondrial DNA from a 27,000-year-old Siberian mammoth. Also this week, a *Nature* paper reports using a souped-up version of more conventional methods to sequence a mammoth's entire mitochondrial genome.

Besides helping reveal the origins of mammoths, the new nuclear data serve as a dramatic demonstration of the power of the new technique to reliably sequence large amounts of ancient DNA, other researchers say. "The 'next generation' sequencer that was used [in the *Science* paper] will revolutionize the field of ancient DNA," predicts evolutionary biologist Blair Hedges of Pennsylvania State University in University Park. Ancient DNA pioneer Svante Pääbo of the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany, who co-led the independent mitochondrial study, calls the nuclear DNA work "really great—the way forward in ancient DNA is to go for the nuclear genome with technologies like this."

To get mammoth samples for the new method, molecular evolutionary geneticist Hendrik Poinar of McMaster University in Hamilton, Canada, took bone cores from woolly mammoths found in permafrost and stored in a frigid Siberian ice cave. When Poinar returned the samples to his lab, he was surprised by the amount of DNA that emerged, particularly from one mammoth jawbone. This specimen had been recovered from the shore of Lake Taimyr, where very cold winters and short, cool, and dry summers turned out to be ideal conditions for preserving DNA.

Poinar sent the DNA-rich sample to genomicist Stephan C. Schuster at Pennsylvania State University, University Park, who is working with a new genome sequencer developed by a team at Stanford University and 454 Life Sciences Corp. of Branford, Connecticut (*Nature*, 15 September, p. 376). This rapid, large-scale sequencing technology sidesteps the need to insert DNA into bacteria before amplifying and sequencing it. Instead, scientists break DNA into small fragments, each attached to a tiny bead and encapsulated by a lipid bubble where the DNA is multiplied into many copies for sequencing. Because each fragment is isolated before copying, the method avoids bias from copying large amounts of contaminant DNA from bacteria or humans.

The researchers were stunned by how well the method worked on ancient DNA, which is notoriously difficult to extract and sequence: "I would have been happy if we got 10,000 bases of mammoth DNA," said Poinar. Instead, they got 28 million basepairs, 13 million from the mammoth itself. Their preliminary analysis shows that the mammoth was a female who shared 98.55% of her DNA with modern African elephants. But mammoths were apparently closest kin to Asian elephants, as shown by Pääbo's mitochondrial study, which retrieved about 17,000 basepairs.

Poinar's team also found sequences from bacteria, fungi, viruses, soil micro-organisms, and plants, which the researchers say will help reconstruct the mammoth's ancient world. The technique was so productive that the authors predict it will be used soon to sequence entire genomes of extinct animals. —ANN GIBBONS

With reporting by Michael Balter.

*www.sciencmag/org/cgi/content/abstract/1123360

ScienceScope

Peach State Sticker Shock

Georgia scientists are worried that a U.S. federal appeals panel might side with Cobb County school officials after the panel heard oral arguments last week on the content of antievolution stickers placed in textbooks.

Georgia Citizens for Integrity in Science Education say that a three-judge panel in Atlanta received "erroneous" information at its 15 December hearing. The court was reviewing a lower court ruling that the stickers, which call evolution "a theory, not a fact," unconstitutionally advance a religious view. The court failed to acknowledge scientific errors in the sticker, the education group laments, and wrongly assumed that the school board acted before fundamentalist parents complained, thus mooting the argument that the stickers were a response to religious influences. The school board disavows any religious motive, saying that the stickers encourage "critical thinking."

—CONSTANCE HOLDEN

Flu Preparedness Dealt Blows

PARIS—Efforts to wield two key weapons against a future H5N1 influenza pandemic have suffered setbacks. Last week, French vaccine maker Sanofi Pasteur announced that a prototype H5N1 vaccine containing aluminum as an "adjuvant," or immune booster, appears to offer protection only when two doses of 30 micrograms of antigen each were given.

Sanofi calls the study "progress," but many researchers are disappointed that the booster didn't allow smaller doses to protect. Because the world's flu vaccine manufacturing capacity is limited, they had hoped that the addition of aluminum might bring the dose needed all the way down to 2 micrograms or less, enabling vaccine makers to make billions of doses. "[A] much better adjuvant is needed," says Albert Osterhaus of Erasmus Medical Center in Rotterdam, the Netherlands.

Meanwhile, in this week's *New England Journal of Medicine*, researchers report having isolated from two Vietnamese patients H5N1 strains that are highly resistant to the drug oseltamivir, stockpiled by rich countries. Before that, only one partially resistant H5N1 strain had been found. An accompanying commentary says the "frightening" results mean that oseltamivir must be used wisely and urges measures to prevent people from hoarding the drug.

—MARTIN ENSERINK

www.who.int/entity/vaccine_research/diseases/influenza/Gerdil.pdf

WHO Meeting on Development and Evaluation of Influenza Pandemic Vaccines

sanofi pasteur France – H5N1 Vaccine

Development and preclinical evaluation

2ND-3RD November 2005

World Health Organization
Institute for Vaccine Research
Global Influenza Programme

Hôtel Warwick, Geneva, Switzerland

Catherine GERDIL

Sanofi Pasteur's continuous role in Influenza Protection and Pandemic Preparedness

- **Supplying 40% of doses distributed annually worldwide; continuing to invest in capacity expansion in-line with global demand**
- **Sanofi Pasteur is undertaking efforts globally in pandemic preparedness**
  - ✓ Through initiatives with the US government
  - ✓ Through initiatives with the Australian government
  - ✓ Through initiatives at the European level, including participation to the FLUPAN project and preparation of the mock up dossier according to EMEA guidelines
  - ✓ Through initiatives with other governments
  - ✓ Trough continuous participation to scientific and policy forums
  - ✓ Through internal contingency working plan to ensure readiness in pandemic situation

sanofi pasteur

The vaccines business of sanofi-aventis Group

Key Considerations : Pandemic vaccine development project goal

Develop a process for a safe and immunogenic pandemic vaccine according to EMEA guidelines

Submit a mock up pandemic vaccine file to EMEA

Using an avian strain vaccine candidate (A/H5N1) (A/Vietnam/1194/NIBRG-14)

- Provided by a WHO reference centre (NIBSC)

Vaxigrip- derived process

Monovalent inactivated split vaccine

Production at large scale in current production facilities of Phase II clinical lots

Documentation of pharmaceutical registration dossier based on large scale production data

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The vaccines business of sanofi-aventis Group

Key Considerations : Major steps of sanofi pasteur plan

2004 working program :

- **Pilot scale production of H5N1 strain :**
  - Obtention of every authorisation for production
  - Vaxigrip process at pilot scale
  - Validation of every decontamination procedure
  - Validation of splitting and inactivation treatment in the process
- **Production of standard antigen**
- **Non clinical safety studies : no side effect reported**
- **Stability studies initiated on monovalent bulks**

Animal preclinical study with H5N1 formulation adjuvanted with alum in rabbits

- **Local tolerance study**
- **Monovalent vaccine : A/H5N1/VN1194/04 x PR8 RG reassortant**
  - IM route
  - Vaccine doses : 0 – 7.5 –15 and 30 µg of HA
  - Alum
- **8 naïve female rabbits / group**
- **3 injections at D0, D14 and D28 by IM route**
- **Blood sampling: D3 ,D14, D31 and D42**
- **Analysis of humoral response by Elisa and IHA**

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The vaccines business of sanofi-aventis Group

Animal preclinical study with H5N1 formulation adjuvanted with alum in rabbits

Results:

- **No mortality, no systemic clinical signs, no changes in body weight gain and food consumption**

- **Local clinical signs: occasional (1 to 2 animals out of 8 per group) transient (1 to 3 days) hematoma. More extended in high dose treated animals. No erythema, nor oedema, nor induration.**

Animal preclinical study with H5N1 formulation adjuvanted with alum in rabbits

Conclusion :

Under the conditions of the study, single or repeated IM injection of 7.5, 15 or 30 µg H5N1 + Alum were clinically well tolerated in the rabbit.

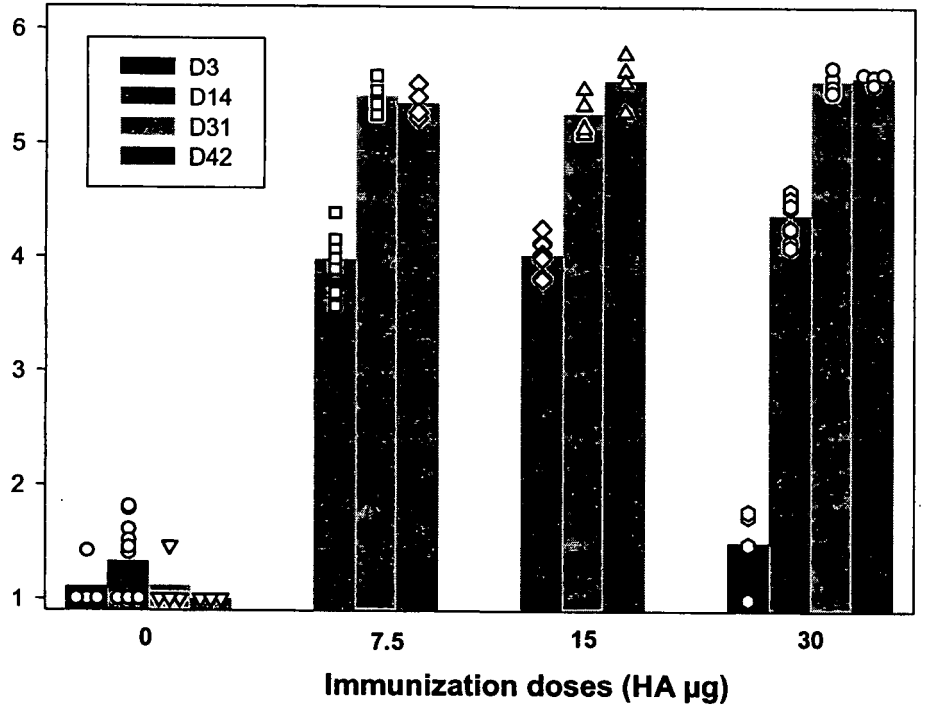
Inflammatory changes seen histopathologically after single or repeated injections were classical observations after aluminum adjuvanted vaccine injections.

In rabbits, high IHA titers are associated with high ELISA titers after 2 immunizations, whatever the immunization dose

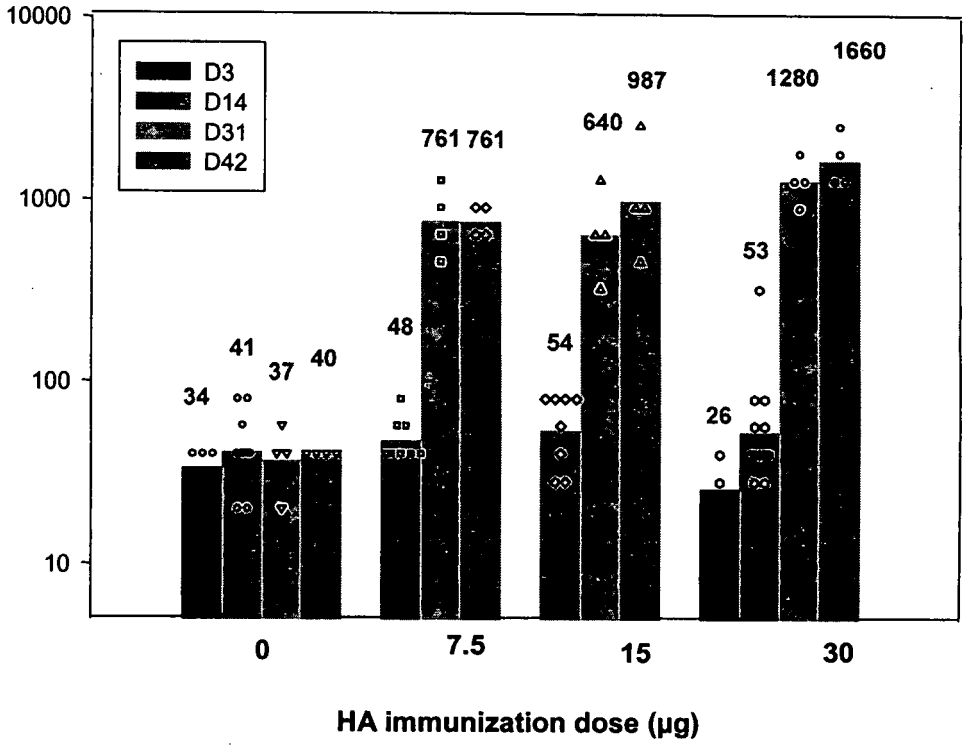
H5N1+ alum formulations

ELISA titers

(log_{10} of H5N1-antibody titers)



IHA titers



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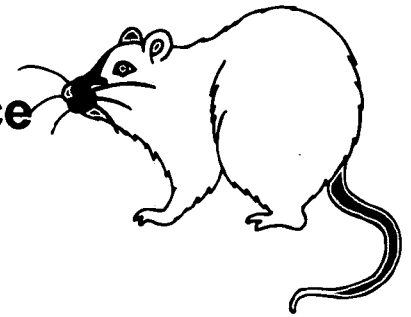
Animal studies performed in BALB/c mice



- Monovalent vaccine : **A/H5N1/VN1194/04 x PR8 RG reassortant**
  - **Non adjuvanted : 0.1 – 0.4 – 1.5 and 6 µg/HA per dose**
  - **Alum adjuvanted : 1.5 µg/HA/dose and Alum (1/10 of human dose)**
- 10 naïve Balb/c mice / group
- 1 or 2 injections by IM route (2 x 50µl)
- Blood sampling: D0, D14 ,D28, D42 , D56, D77 and D105
- Analysis of humoral response by Elisa, IHA and SN

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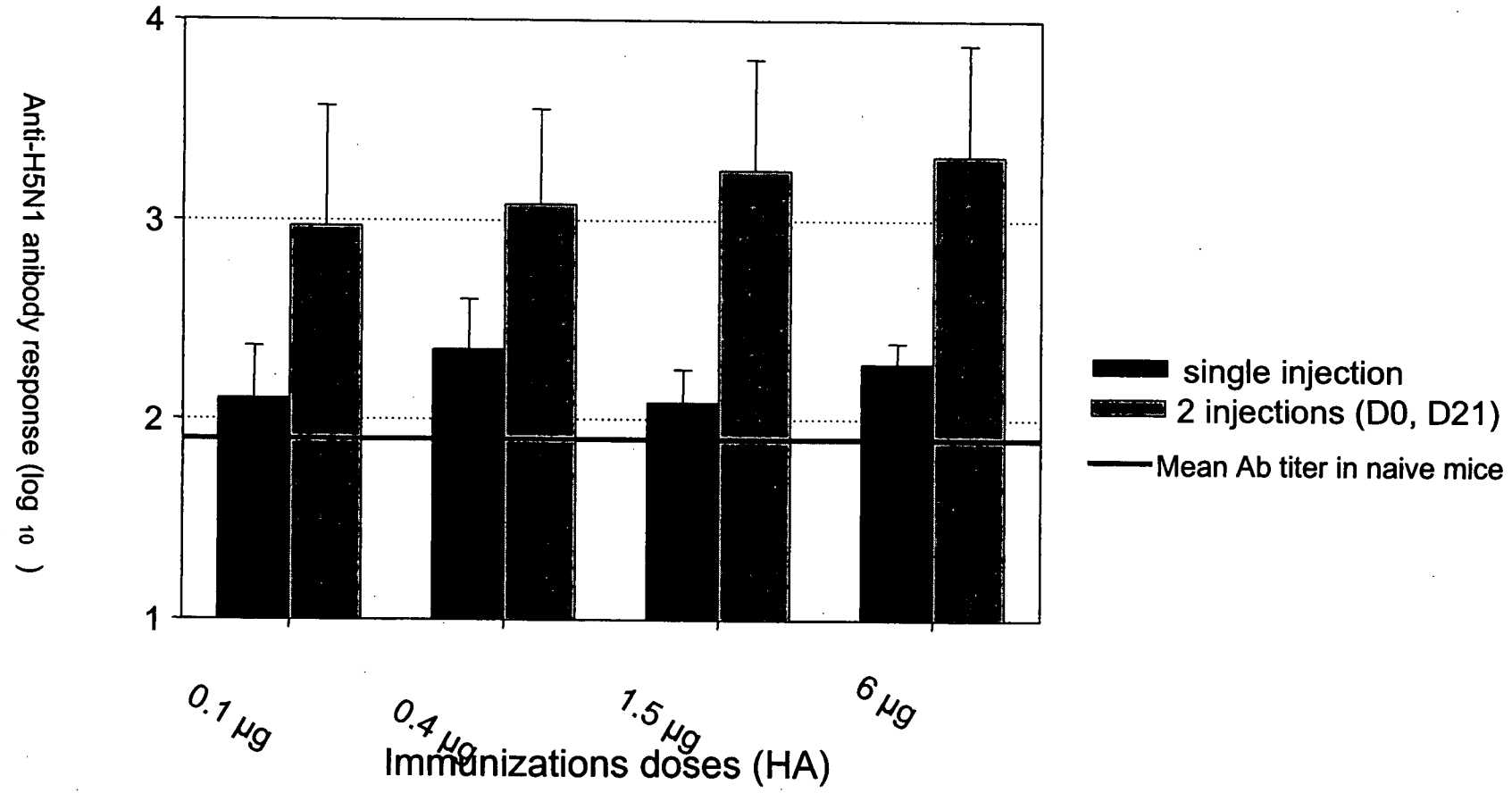
The unadjuvanted H5N1 vaccine is poorly immunogenic in Balb/c mice → a low antibody response is measured by anti-H5N1 ELISA (D28)

Anti-H5N1 antibodies measured at D28 in mice immunized with A/H5N1



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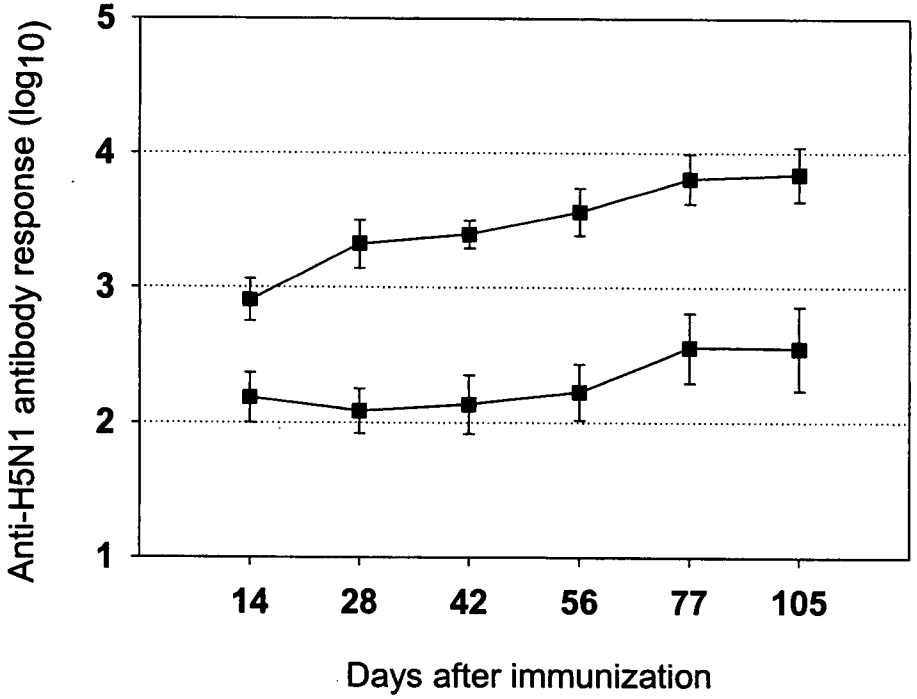
Administration of A/H5N1 formulated with Alum elicits a higher antibody response than unformulated vaccine

Immunization with **1.5 µg of HA (1/10 human dose)** with :

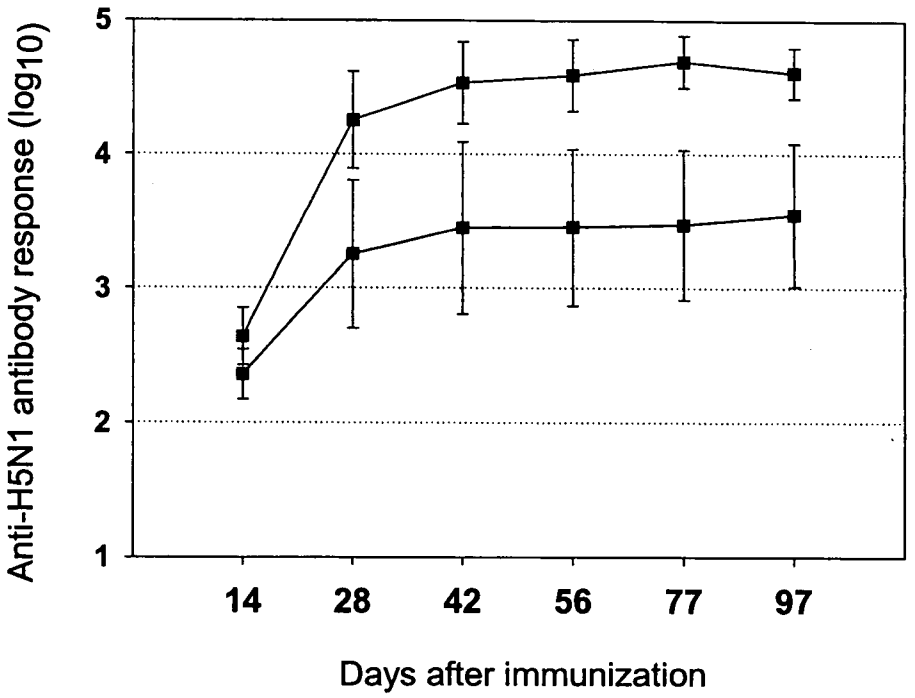
No adjuvant

Alum

Single injection, D0



Two injections, D0 and D21



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Concerns regarding dettection of functional antibodies against the H5N1/VN/1194/04 strain

Already described in the litterature

Odagiri *et al* (Malta meeting, Sept 2005, oral presentation,)

- In Balb/c mice immunized with monovalent H5N1 (whole virus, VN1194/04 strain) administered in Alum → no HAI nor SN detectable BUT protection from a viral challenge
- With HK/2003 strain : good correlation betwen IHA titers and protection

Webster *et al* (PNAS, July 2005)

- HAI titers are generally low or undetectable in ferrets immunized with VN/2004 strains compared to HK/2003 strains → related to a specific HA residue N223 that increases the sensitivity of the HI assay

Key Considerations : 2005 sanofi pasteur plan

- **2005 working program :**
  - **2005 Phase 1 Clinical study conducted with the expected timing :**

    Randomized, open, multicentric study in France

    300 healthy adults aged from 18 to 40 years / 50 subjects per arms

    3 formulations with and without alum adjuvant – Mix and shoot study.

    2 doses, three weeks apart

  - **Objective of the study :**

    Immunogenicity response after each vaccination

    Safety profile during the 21 days following each injection

    Anti-HA Ab persistence 6 and 12 months after the first vaccination

  - **Results :**

    → No adverse events reported

    → Serology still on going - Results expected by the end of 2005

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The vaccines business of sanofi-aventis Group

Key Considerations : 2005-2006 sanofi pasteur plan

- **2005 large scale production implementation : A large experience has been aquired**
  - Good collaboration between experts, manufacturers and WHO biosafety expert group for review of pandemic vaccine production guidelines
  - Collaboration between authorities and sanofi pasteur for large scale production implementation
  - Enhanced BSL2 confinment implemented with specific personnal education and protection
  - Validation of decontamination procedures using H5N1 modele
  - Vaxigrip process used → Monovalent batches representative from current vaccine production
- **Stability studies :**
  - Performed on monovalent bulks produced at large scale .
  - Good stability of monovalent bulks reported after 6 months at +5°C . (pilot scale production)
- **Ready to use formulation including adjuvant under development**
- **Formulation of phase II clinical study batches using the selected formulation for large scale pivotal study to be conducted in 2006**
- **Documentation of large scale pandemic vaccine production for EMEA mock up registration program dossier.**

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Key Considerations : Lessons learned from 2004 - 2005 preparedness project experience

- **Implementation of the production process :**
  - Complexity of the process : Number of authorisations to be obtained before any manipulation of the strains:...
  - Large experience regarding large scale production of avian RG modified strains taking into account European GMO regulation

- **Strain characteristics**
  - Low density of the virus
  - Dissociation between infectious titer and UHA in allantoic fluid

- **Product and Process qualification :**
  - Development of reagents : difficulty to obtain hyperimmunised ferret sera
  - Development of SRD reagents in collaboration witth NIBSC and CBER
  - Development and validation of QC testings adapted to this new strain

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PAP-985

Journal of Biological Standardization 1977 5, 237–247

V

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An improved single-radial-immunodiffusion technique for the assay of influenza haemagglutinin antigen: application for potency determinations of inactivated whole virus and subunit vaccines*

J. M. Wood,† G. C. Schild,† R. W. Newman,† and Valerie Seagroatt,†

An improved single-radial-diffusion technique for the assay of influenza haemagglutinin antigen is described. The modified method enables the results of assays of antigen to be obtained more rapidly and with greater precision than previously. The use of immunoplates containing varied, pre-selected concentrations of anti-haemagglutinin antibody allows accurate assays to be performed over a wide range of antigen concentrations. Concentrations of haemagglutinin as low as 40 i.u./ml could be assayed with accuracy and reproducibility using immunoplates containing low antibody levels. The method is applicable to the accurate determination of haemagglutinin concentrations over the ranges likely to be present in inactivated influenza vaccines.

In tests on 'whole virus' antigen preparations, it was found that the ratio between haemagglutination titre (i.u./ml) and haemagglutinin antigen activity (μg/ml) determined by single-radial-diffusion was relatively constant for antigens containing a given strain but showed variation between strains (range 16·5–26·8 i.u./μg HA activity). For the subunit vaccines examined this ratio showed a large degree of variation (range 1·4–16·6 i.u./μg HA activity) and in general was considerably lower than for whole virus antigens.

These findings support the conclusion that techniques based on the agglutination of erythrocytes may provide data on vaccine potency which are not directly comparable from strain to strain for 'whole virus' vaccines and that these methods are entirely inappropriate to potency assays of split-product or subunit vaccines. In contrast, single-radial-diffusion may be of value for assays of both 'whole virus' vaccines and those containing disrupted virions.

* Received for publication 8 February 1977.

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INTRODUCTION

The single-radial-diffusion (SRD) technique has been proposed as a possible alternative to tests based on the agglutination of erythrocytes for the assay of the haemagglutinin (HA) antigen content of influenza virus vaccines (Schild, Wood & Newman, 1975). In the present paper we describe an improved modification of the method described earlier by Schild *et al.* (1975) which enables greater accuracy and reproducibility and also our experience with the use of this method for potency assays of 'whole virus' and subunit vaccines.

Although there is considerable interest in the development of split-product (Davenport, Hennessy, Brandon, Webster, Barrett & Lease, 1964) and subunit influenza vaccines containing purified HA and neuraminidase (NA) antigens (Bachmayer, Liehl and Schmidt, 1976; Brady & Furminger, 1976; Laver & Webster, 1976) appropriate tests for potency assays of these products have not been defined. The studies to compare SRD with traditional methods for potency assays of influenza vaccines described in this paper provide evidence that assays based on haemagglutination are not appropriate for subunit vaccines. However, SRD tests appear to be of value for potency assays of these products.

MATERIALS AND METHODS

Preparation of SRD plates

Glass plates (12×12 cm^2) are precoated with an agarose film (1·5% A37 agarose in phosphate-buffered saline, Schild *et al.*, 1975) and dried. A Perspex mould (Fig. 1)

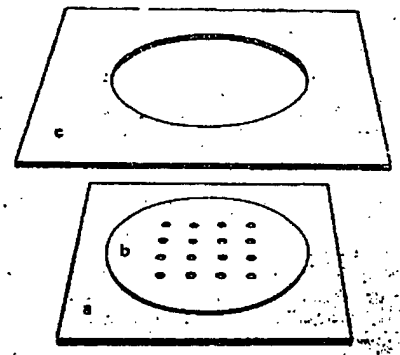

Fig. 1. 12 × 12 cm glass plate (a) precoated with agarose upon which is cast a disc of agarose 9 cm in diameter (b) using a Perspex mould (c). Wells, 4 mm in diameter, are cut in the agarose using a Perspex template.

with internal diameter 9 cm is sealed to the glass plate using a few drops of molten agarose. After the sealing gel has solidified, 12 ml of agarose containing an appropriate concentration of anti-HA serum is poured into the mould. The gel is allowed to set (approximately 15 min) and several wells, 4 mm in diameter, are punched into the agarose using a Perspex template and the mould is removed.

Performance of SRD tests

Volumes of 20 µl of appropriate dilutions of reference and test antigens are introduced into the wells in the agarose followed 15 min later by 10 µl volumes of detergent, sodium

lauryl sarcosinate (sodium sarcosyl, Ciba-Geigy NL97, 5% in H_2O), as previously described (Schild *et al.*, 1975). It is important that each dilution of the test and reference antigen is prepared independently in order to obtain a valid dose–response curve. It is recommended that tests be performed on duplicate SRD plates and that the position of the test and reference antigens be randomized on the plates.

Processing and reading of SRD plates

After leaving the plates for 24 h in a moist chamber to allow diffusion of the antigen, they are washed for 30 min in phosphate-buffered saline and the gel is then pressed and dried according to the method of Axelsen, Kroll & Weeke (1973). A sheet of moist filter paper is laid on the agarose surface, followed by four layers of absorbent lint and covered by a sheet of glass. A weight of 650 g (10 g/cm^2 of agarose) is placed on the glass. The gel is pressed for 15 min and then for a further 15 min after the lint is changed. The weight and lint are removed and the gels, with filter paper attached, are allowed to dry in warm air. When perfectly dry, the filter paper is peeled off and the plates are stained in Coomassie Brilliant Blue (0·3% w/v in a mixture of 12% acetic acid and 29% methanol) for 5–10 min. The plates are then destained in two changes of methanol–acetic acid mixture (12% acetic acid, 29% methanol) for 15–30 min, until clearly defined stained zones are visible, and finally dried in warm air.

The diameter of the stained zones surrounding antigen wells is measured in two directions at right angles using a micrometer eyepiece, dose–response curves of antigen dilution against zone annulus area are constructed and the results are calculated according to standard slope-ratio assay methods (Finney, 1952).

Guidelines for specificity and potency of anti-haemagglutinin sera for use in SRD assays

Suitable antisera may be prepared by the immunization of goats or rabbits with purified HA subunits together with Freund's complete adjuvent. Appropriate techniques for the isolation of HA from purified virus include the treatment of virus with bromelain (Brand & Skehel, 1972) followed by the separation of HA on sucrose velocity gradients or by the separation of HA from detergent-disrupted virus by electrophoresis on cellulose acetate strips (Laver, 1964; Schild, 1970). Considerable variation exists in the behaviour of different virus strains to treatment with bromelain or detergents and the best method must be established for each strain by experimentation.

It is important that the reference hyperimmune anti-HA sera should be subjected to tests for specificity and potency in order to establish their usefulness for SRD assays.

(1) The purified HA used as antigen should be shown by polyacrylamide gel electrophoresis to consist of two polypeptide species (HA1 and HA2) with molecular weights of approximately 58 and 28×10^3 (Skehel & Schild, 1971).

(2) Antisera should be tested for specificity to HA by appropriate techniques (see review: Schild & Dowdle, 1975) including haemagglutination-inhibition, neuraminidase inhibition, SRD and immuno-double-diffusion (IDD). Plate 1 illustrates IDD tests to confirm the specificity of the reference antiserum to A/Victoria/3/75 HA described below. There should be no detectable antibody to neuraminidase, nucleoprotein or matrix protein in the serum.

(3) Antisera should show the expected degree of cross-reactivity and spur formation when tested in IDD against homologous HA antigen and strains possessing antigenic variation within the same subtype (Plate 1).

(4) The potency of anti-HA antisera may be determined by an SRD assay for antibody using purified homologous virus in an agarose gel under standard conditions using 0·2 mg virus antigen/ml gel (Schild, Aymard-Henry & Pereira, 1972). In order to be useful as an SRD reference reagent, antisera should produce zones of at least 20 mm².

Reference reagents 76/547 *and* 76/548

Freeze-dried reference reagents are now available from the National Institute for Biological Standards and Control (N.I.B.S.C.) for SRD assay of the HA of A/Victoria/3/75 (H3N2) virus antigen. The reference antiserum 76/547 was prepared in goats to the purified HA of A/Victoria/3/75 virus, X47 strain. X47 is a recombinant between A/PR8/34 (HON1) and A/Victoria/3/75 (H3N2) prepared by Dr E. D. Kilbourne, Mount Sinai Hospital, New York. It contains HA (H3) and neuraminidase (N2) antigens derived from A/Victoria/3/75 and possesses the high yielding growth characteristics of A/PR8/34. The HA antigen was extracted from purified X47 virus by treatment with bromelain. Two doses of approximately 300 μg of HA with Freund's complete adjuvant were given intramuscularly at 3 week intervals and serum was collected 10 days after the second immunization. The serum produced zone areas of approximately 70 mm² in SRD tests using purified X47 virus in the gel and had an HI titre of 1 : 30 000.

The serum was shown to be specific to the HA of A/Victoria/3/75 by immuno-double-diffusion and SRD (Plate 1). Each ampoule of freeze-dried serum (76/547) contains 1 ml of a one-eighth dilution of the goat serum. When the contents of one ampoule are added to 24 ml agarose (sufficient agarose for two SRD plates), the serum concentration is 5 μl serum/ml agarose.

Reference antigen 76/548 is a preparation of A/Victoria/3/75 virus, X47 strain which has been purified by rate zonal centrifugation and the HA antigen activity calibrated in SRD tests using highly purified X47 virus of known protein content (Lowry, Rosebrough, Farr & Randall, 1951).

TABLE 1. Calibration of HA antigen activity of reference antigens 76/548 and 74/560

Batch of purified virus		Concentration of haemagglutinin: Purified virus* (μg/ml)	Reference antigen (μg activity/ml): 76/548†	74/560‡
X47	1	2280	34·5	
	2	1770	41·4	
Mean			37·9	
MRC11	1	1800	—	81·8
	2	2970		83·3
	3	3180		80·4
	4	2700		74·5
Mean				80·0

* Lowry protein estimate.
† Estimate by assay of 76/548 and purified X47 virus in SRD tests.
‡ Estimate by assay of 74/560 and purified MRC11 virus in SRD tests.

Reference 76/548 was estimated to contain 38 μg of HA activity which is the mean of two determinations using separate batches of X47 virus (Table 1); this is equivalent to 1000 i.u. for the A/Victoria/3/75 virus strain.

Similarly, reference A/Port Chalmers/1/73 antigen, 74/560 was calibrated with four separate batches of MRC11 virus (recombinant with antigenic characters of A/Port Chalmers/1/73 (H3N2) and high growth character of A/PR8/34 (HON1) using the modified SRD technique (Table 1). Reference 74/560 contains 80·0 μg of HA activity/ml which is equivalent to 1962 i.u./ml for the A/Port Chalmers/1/73 strain of virus.

Reference reagents for assay of the HA's of A/New Jersey/8/76 (Hsw1N1) virus are available from N.I.B.S.C. and are in preparation for B/Hong Kong/8/73 virus.

RESULTS

The basis of SRD assays for HA antigen is the comparison of zone sizes produced by dilutions of a reference antigen and a test antigen (e.g. a vaccine) on the same immunoplate. Thus it is important to establish the homogeneity of reactions within one plate.

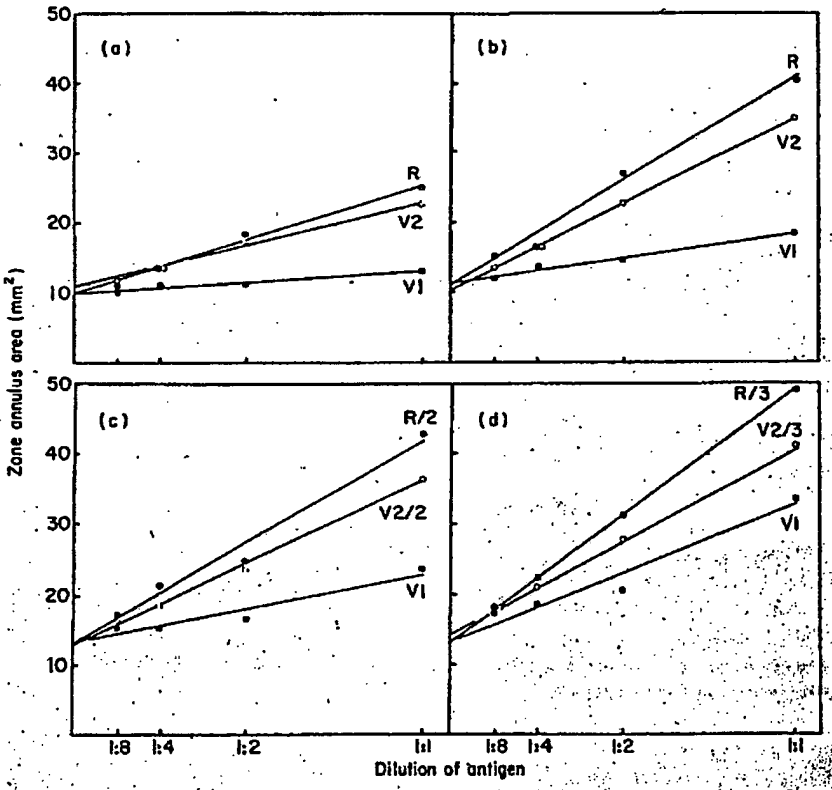

Fig. 2. Dose–response linear regression lines for reference antigen (R), vaccine of 100 i.u./0·5 ml (VI) and vaccine of 400 i.u./0·5 ml (V2) when serum concentration in the agarose is varied, (a) 40 μl 76/547 serum/ml gel; (b) 20 μl/ml; (c) 10 μl/ml; (d) 5 μl/ml. R and V2 are prediluted in tests with low serum concentrations: one-half dilution for 10 μl serum/ml gel, one-third dilution for 5 μl serum/ml gel.

Plate 2 shows a representative immunoplate on which replicate tests were made of a single antigen preparation over the surface of the plate. The variation of the zone sizes throughout the plate did not exceed 5%.

Plate 3 illustrates stained immunoplates containing 20, 10 and 5 μl of anti-HA serum/ml agarose, on which had been tested dilutions of four A/Victoria/3/75 antigen preparations, a calibrated reference antigen (R), two vaccine preparations of differing potency (V1 and V2) and a sample of purified X47 virus (C). With all concentrations of serum the zones were clearly defined and easily measurable. There was an inverse relationship between antiserum concentration and zone size. With the lowest concentration of antiserum a vaccine of low potency (nominally 100 i.u./0·5 ml) gave zones large enough to be accurately measured (8 mm diam.).

Dose–response curves for antigens R, V1 and V2 on plates containing a range of antiserum concentrations from 40 to 5 μl/ml agarose are shown in Fig. 2. Since the more potent antigens (R and V2) gave very large zones on plates containing low antiserum concentrations these were pre-diluted before preparing serial dilutions for test (one half for tests using 10 μl serum/ml agarose and one-third for tests using 5 μl serum/ml agarose). For each concentration of antiserum there was a linear relationship between dilution of antigen and zone annulus area as found in previous studies (Schild *et al.*, 1975). When the regression lines were fitted with a common intercept, the slopes of the lines were proportional to the potency of the antigen (Finney, 1952).

It is seen (Table 2) that the estimated potencies of the two test antigens (V1 and V2) are in good agreement over the range of serum concentrations used.

TABLE 2. Effect of serum concentration in agarose on estimate of vaccine potency by SRD

	Calculated antigen concentration*			
	Vaccine 2		Vaccine 1	
Concentration of serum (μl/ml agarose)	μg HA activity/0·5 ml†	i.u./0·5 ml	μg HA activity/0·5 ml†	i.u./0·5 m
40	14·5	380	3·69	97
20	14·8	389	3·55	93
10	13·8	362	3·02	79
5	14·4	378	3·67	96
Mean	14·3	377	3·48	91
95% confidence limits of mean	13·7–15·0	359–395	2·98–3·97	78–104

* Calculated using reference antigen 76/548 (38 μg HA activity/ml, 1000 i.u./ml.
† Results are expressed in terms of 0·5 ml volumes since this is the conventional dose for an inactivated vaccine.

The reproducibility of the technique using low serum concentrations (5 μl/ml agarose) was assessed over a 3 day period. Two vaccines of low antigenic content were assayed against the reference antigen 76/548, pre-diluted one-third. The antigen dilutions were randomized on duplicate plates and the results were analysed as a randomized block. All three assays were statistically valid at the 5% level using the criteria of common intercept and insignificant deviation from linearity. The potency estimates with

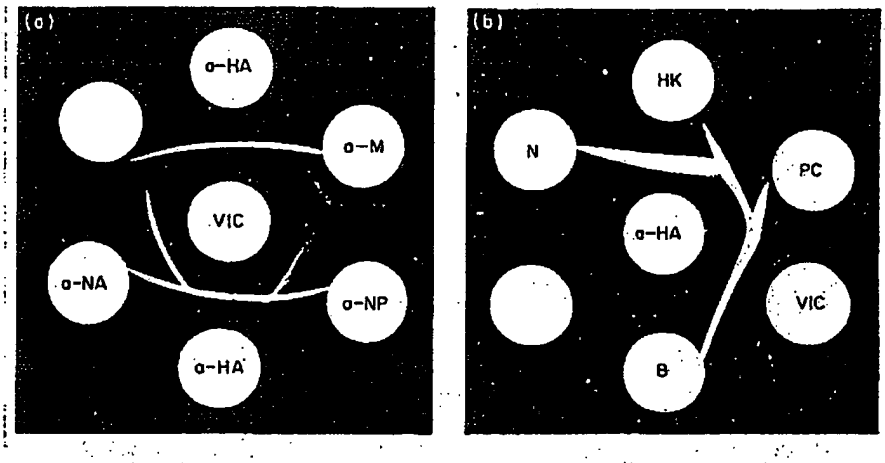

Plate 1. Use of immuno-double-diffusion tests for determination of specificity of reference antiserum to A/Victoria/3/75 HA (ref. 76/547). Wells marked VIC, HK and PC were filled with concentrated virus preparation of A/Victoria/3/75 (H3N2) (X47 strain), A/Hong Kong/1/68 (H3W2) and A/Port Chalmers/1/73 (H3N2). Wells marked N and B contained avian influenza virus A/Chicken Germany/N/49 (Hav2Neql) and B/Hong Kong/8/73. The virus antigens were disrupted in the wells by the addition of sodium sarcosyl detergent.

Wells marked a-HA, a-M, a-NP and a-NA were filled with undiluted hyperimmune sera as follows: a-HA, goat antiserum to purified A/Victoria/3/75 HA (ref. 76/547); a-M, rabbit antiserum to purified influenza A matrix protein; a-NP, rabbit antiserum to purified influenza A nucleoprotein; a-NA, rabbit antiserum to purified neuraminidase of A/Port Chalmers/1/73 virus. Plate 1(a) shows that the precipitin reaction given by the reference antiserum for A/Victoria/3/75 HA is composed of a single clearly defined line showing lack of identity with lines produced by reference antisera to other virus components (neuraminidase-N2, nucleoprotein and matrix protein antigens).

Plate 1(b) shows that the antiserum to A/Victoria/3 HA cross-reacts with viruses containing related H3 haemagglutinins, A/Hong Kong/1/68 and A/Port Chalmers/1/73. The spurs between the reactions given by the different strains indicate that antigenic differences exist between the HA antigens of these strains and the homologous A/Victoria/3/75 virus, whilst the shared reactions confirm that all antigens are of the H3 subtype. Similar patterns of cross-reaction have been reported previously (Schild *et al.*, 1973). Virus strains containing unrelated HA antigens (influenza B/Hong Kong/8/73 and A/Chicken Germany/N/49) gave no precipitin reactions.

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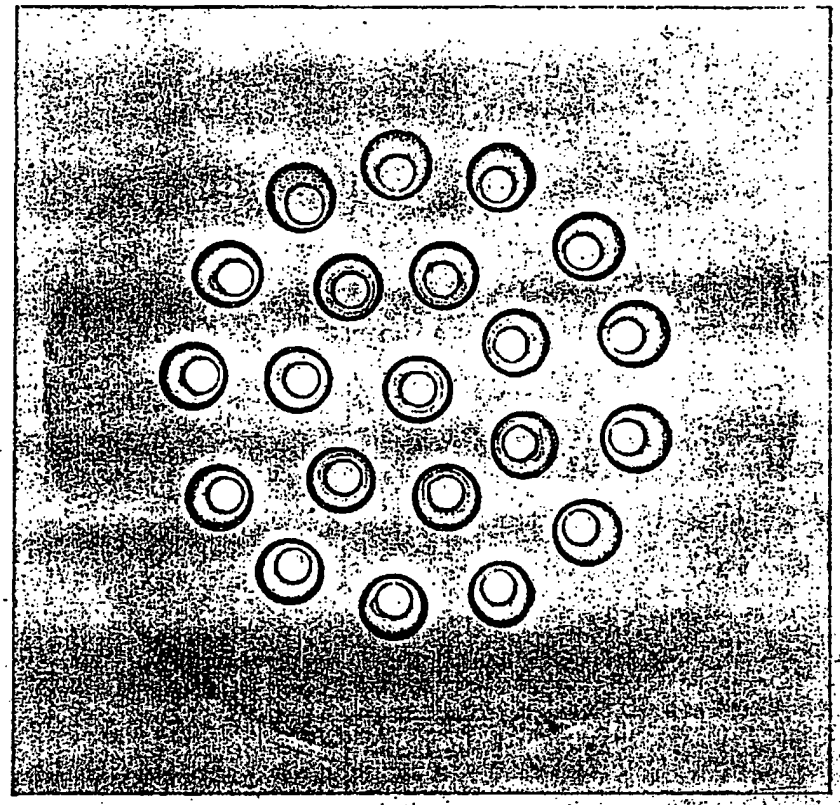

Plate 2. SRD test showing uniformity of diffusion zones across glass plates. Gel contains 76/547 antiserum (5 µl/ml agarose), wells contain 20 µl amounts of antigen 76/548.

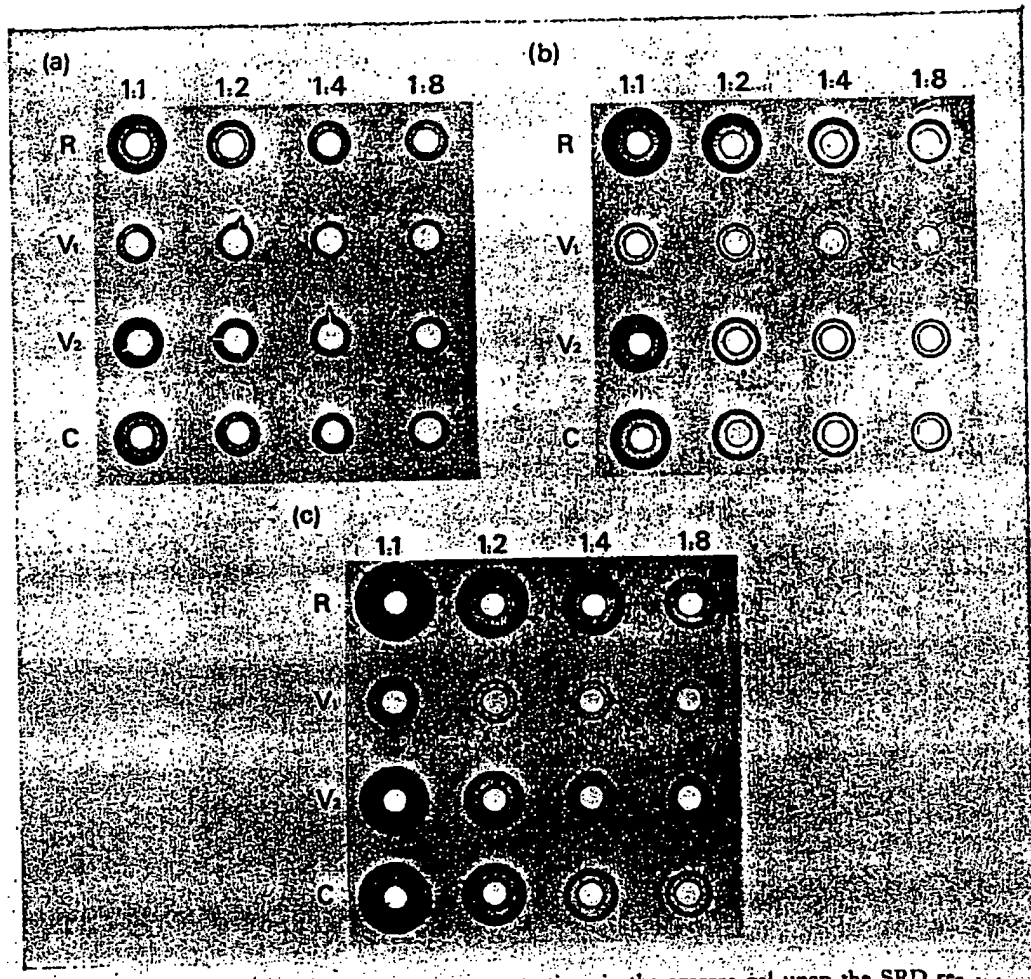

Plate 3. Effect of different serum concentrations in the agarose gel upon the SRD reaction zones. The gels contain (a) 20 µl 76/547 serum/ml gel, (b) 10 µl/ml, (c) 5 µl/ml. The wells in rows R, VI, V2 and C contain reference antigen 76/548, vaccine of 100 i.u./0·5 ml, vaccine of 400 i.u./0·5 ml and concentrated purified X47 virus respectively.

TABLE 3. Reproducibility of SRD test using low serum concentrations in agarose

		Calculated antigen concentration*			
				95% confidence limits	
	Day of assay	μg HA activity/0·5 ml	i.u./0·5 ml	μg HA activity	i.u.
Vaccine A	1	3·8	100	3·3–4·3	88–113
	2	3·5	92	3·2–3·8	85–100
	3	3·8	100	3·5–4·1	91–107
	Mean	3·6	96	3·5–3·8	92–101
Vaccine B	1	3·8	100	3·3–4·2	88–112
	2	3·5	93	3·2–3·8	85–100
	3	3·7	97	3·4–3·9	89–104
Mean		3·6	96	3·5–3·8	91–100

* Calculated using reference antigen 76/548.

TABLE 4. Ratios between haemagglutinating activity (i.u./ml) and HA concentration (μg/ml) estimated by SRD for different influenza virus strains

		Potency estimates		
Influenza virus strain	Antigen preparation	i.u./ml*	μg HA activity/ml assessed by SRD†	Ratio i.u./μg HA activity
A/Port Chalmers/1/73 (H3N2) (MRC11 strain)	Whole virus‡	1962	80·0	24·5
		1062	39·6	26·8
		500	21·4	23·4
	Subunit§	7660	460·9	16·6
		1129	510·1	2·2
A/Victoria/3/75 (H3N2) (X47 strain)	Whole virus	1780	78·0	22·8
		651	30·8	21·1
	Subunit	4463	58·3	7·6
A/New Jersey/8/76 (Hsw1N1) (X53 strain)	Whole virus	900	54·5	16·5
		2347	136·8	17·1
	Subunit	1214	847·0	1·4
B/Hong Kong/8/73	Whole virus	900	36·0	25·0

* i.u. assays performed by haemagglutination tests as described by Westwood *et al.* (1971).
† Data obtained by use of reference antigen calibrated against purified virus of known HA content.
‡ Inactivated monovalent 'whole virus' vaccines found by electron microscopy to contain mainly intact virus.
§ Purified subunit vaccines (Brady & Furminger, 1976).

their 95% confidence limits are shown in Table 3. These estimates were judged to be homogeneous by the χ^2 test, indicating that the reproducibility of the assay method was good.

In addition to the studies with A/Victoria/3/75 virus described above, the SRD technique has been applied to other influenza A viruses including A/Port Chalmers/1/73 (H3N2), A/New Jersey/8/76 (Hsw1N1) and B/Hong Kong/8/73 using homologous anti-HA sera. These viruses were in the form of inactivated monovalent 'whole virus' vaccines produced commercially by zonal centrifugation and subunit vaccine preparations (Brady & Furminger, 1976). Potency estimates were made for the vaccines in terms of i.u. values (haemagglutination assays; Westwood, Woodward & Perkins, 1971) and in terms of μg HA activity/ml (SRD assays). The ratio of i.u./μg HA activity was calculated for each preparation (Table 4). It can be seen that this ratio was consistent for whole-virus antigens of a given strain and less constant between strains. The subunit antigens examined were commercial bulk materials prepared as described by Brady and Furminger (1976) and tested before dilution to final vaccine strength. The ratios of i.u./μg HA activity varied widely for these preparations and were considerably lower than those obtained for 'whole virus' preparations of the same strains.

DISCUSSION

The modified SRD technique described in the present paper has a number of technical advantages over the method previously described by Schild *et al.* (1975). These include greater precision and reproducibility due to the small degree of within-plate variation in the size of reaction zones; the applicability of the method to the assay of low concentrations of HA, which is possible because of the drying of agarose gels during processing; and the greater rapidity in processing, enabling assays to be performed within 27 h. The modified technique permits work with low concentrations of anti-HA serum, allowing accurate and reproducible quantitation of HA antigen activity as low as 2 μg/ml (approximately 40 i.u./ml). Whilst amounts of antigen considerably below this concentration are detectable, they cannot be accurately measured. The optimal concentration of antiserum for use in the test described varies with the potency of the antigen being assayed. It is recommended that, in order to assay A/Victoria/3/75 vaccines of conventional potency (nominally 400 i.u./0·5 ml), 250 μl of reference anti-HA serum 76/547 should be added to 12 ml of agarose. For vaccines containing low concentrations of HA (100 i.u./0·5 ml) 63 μl of antiserum per 12 ml of agarose should be used. The data presented in Table 2 show that the potency estimates of the reference or test antigens are independent of antiserum concentration.

If the SRD test is to be employed widely for potency assays it will be necessary to develop an internationally accepted unit of measurement for the HA content of influenza vaccines based on this test. One possible unit of measurement is μg HA activity/ml (Schild *et al.*, 1975). Determination of HA concentrations may be achieved by calibrating working reference antigen preparations in terms of μg HA activity/ml using highly purified influenza virus containing known amounts of HA. The basis of this estimation is the knowledge of the polypeptide composition of the purified influenza virus preparation and the determination of the HA protein as a proportion of total virus protein. In our studies this value was taken as 33% from the determination for

A/Hong Kong/68 (H3N2) virus by Skehel & Schild (1971). The viruses included in the present studies were shown by polyacrylamide gel analysis not to differ significantly in polypeptide composition from A/Hong Kong/68 virus. For routine purposes, a working reference antigen is calibrated with a minimum of two or three different preparations of purified virus, and the values for μg HA activity/ml should be consistent for each of these preparations (variation $\leqslant 20\%$), before a mean value is ascribed to the working reference.

The SRD technique also allows vaccine potency to be expressed in terms of i.u. equivalents by comparing the SRD reaction of a test antigen with that of a reference preparation which has been calibrated in i.u. by haemagglutination titrations against an HA standard (Krag & Weis Bentzon, 1971; Westwood *et al.*, 1971).

The data in Table 4, showing the ratios between the potencies of different antigens when determined by standard haemagglutination titration (i.u.) and by SRD, emphasize the need for a suitable replacement for potency tests of inactivated influenza vaccines based on the agglutination of erythrocytes. Although the relationship between haemagglutination titre and μg HA activity/ml was reasonably constant for 'whole virus' preparations within a given strain of virus, the ratio varied when different virus strains were examined (16·5–26·8 i.u./μg HA activity). There was even greater variation in the ratio between different subunit vaccine preparations (1·4–16·6 i.u./μg HA activity). The lowest ratio was detected for swine influenza (A/New Jersey/76) vaccines. Thus, based on equivalent haemagglutinin activities, vaccines containing this strain would be expected to contain more haemagglutinin antigen than vaccines containing A/Port Chalmers/73 or A/Victoria/3/75 viruses. It may be assumed that the haemagglutination titre of an antigen is a function of the intrinsic properties of the HA of the virus strain (binding affinity for erythrocytes) and the physical state of the antigen in the preparation. For 'whole virus' vaccines the degree of aggregation of virus particles, the morphology of particles (spherical or filamentous) and the presence of partially disrupted virus are relevant factors. For split-product or subunit vaccines the ratio would be expected to differ from that of 'whole virus' preparations and would depend on the highly variable arrangement of HA subunits in the preparation. For the subunit vaccines tested the haemagglutination titre was low in comparison to the amount of HA antigen activity present. Thus conventional haemagglutination assays would underestimate the potency of these preparations. In the extreme case, certain methods of HA antigen preparation may result in mainly monomeric subunits which are devoid of haemagglutination activity but they may contain large amounts of antigen detectable by SRD.

Assays based on SRD are not dependent on the physical state of the antigen since the addition of sodium sarcosyl detergent results in the production of diffusible HA subunits whatever the original state of the antigen.

A further advantage of the SRD technique is that it may be applied to each of the HA components in the final product of multivalent influenza vaccines containing two or more virus types or subtypes, by means of a range of immunoplates containing appropriate anti-HA sera. No cross-reaction between the HA antigen of different influenza types or subtypes has been observed in SRD tests. In contrast, assays based on agglutination measure the entire HA content without selectivity, the amount of each component in the final product being assessed before blending.

In SRD tests with antigenic variant HA antigens it has been found that variant strains within an HA subtype (e.g. H3) produce reaction zones which are clearly distinguished by their much lower intensity than zones produced by the homologous antigen (Schild

& Wood, in preparation). The SRD technique may thus be used effectively to confirm the correct antigenic composition of test vaccines.

It should be emphasized that no *in vitro* technique so far proposed for potency estimation will predict exactly the antibody response to inactivated influenza vaccines in man. The antibody response is dependent on several factors including the physical arrangement of the antigen ('whole virus' or subunit preparations) and the age and existence in vaccine recipients of antigenic memory to antigens identical or related to those in the vaccine (Virelizier, Allison & Schild, 1974). The role of such variables must be determined by experimentation and experience. However, the problem of assessing the amount of antigen in a vaccine is a basic and essential requirement to the determination of appropriate dosage schedules for effective immunization.

The results reported here emphasize the unsuitability of potency estimates based on haemagglutination phenomena for influenza vaccines.

Using similar SRD techniques to those described for HA, estimates of the amounts of other virus-specific antigens (neuraminidase, nucleoprotein and matrix protein) may be obtained. Work on the development of reference reagents for standardization of these other components is currently in progress.

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